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Development of a Simple and Robust Kinetic Model for the Production of Succinic Acid from Glucose Depending on Different Operating Conditions

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Abstract: Succinic acid (SA) is one of the main identified biomass-derived chemical building blocks. In this work we approach the study of its production by *Actinobacillus succinogenes* DSM 22257 from glucose, focusing on the development and application of a simple kinetic model capable of representing the evolution of the process over time for a great diversity of process variables key to the production of this platform bio-based chemical: initial biomass concentration, yeast extract concentration, agitation speed, and carbon dioxide flow rate. All these variables were studied experimentally, determining the values of key fermentation parameters: titer (23.8–39.7 $g \cdot L^{-1}$), yield (0.59–0.72 $g_{SA} \cdot g_{glu}{}^{-1}$), productivity (0.48–0.96 $g_{SA} \cdot L^{-1} \cdot h^{-1}$), and selectivity (0.61–0.69 $g_{SA} \cdot g_{glu}{}^{-1}$). Even with this wide diversity of operational conditions, a non-structured and non-segregated kinetic model was suitable for fitting to experimental data with high accuracy, considering the values of the goodness-of-fit statistical parameters. This model is based on the logistic equation for biomass growth and on potential kinetic equations to describe the evolution of SA and the sum of by-products as production events that are not associated with biomass growth. The application of the kinetic model to diverse operational conditions sheds light on their effect on SA production. It seems that nitrogen stress is a good condition for SA titer and selectivity, there is an optimal inoculum mass for this purpose, and hydrodynamic stress starts at 300 r.p.m. in the experimental set-up employed. Due to its practical importance, and to validate the developed kinetic model, a fed-batch fermentation was also carried out, verifying the goodness of the model proposed via the process simulation (stage or cycle 1) and application to further cycles of the fed-batch operation. The results showed that biomass inactivation started at cycle 3 after a grace period in cycle 2.

Keywords: succinic acid; fermentation; kinetic model; operational conditions; carbon dioxide

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1. Introduction

The growing concern about the effects of climate change and the depletion of fossil resource reserves have driven a movement focused on the use of new renewable energy sources and bio-based chemicals [1]. One of the most promising alternatives is the development of biorefineries, where biomass will be sustainably processed into bioproducts (materials and chemicals) and bioenergy (biofuels, electricity, and heat) [2].

Within the top 12 high-value-added chemicals from biomass, according to the US Department of Energy (DOE) [3], succinic acid is considered one of the key carboxylic acids for the bioeconomy era. Although this compound is widely used in the production of polyesters, polyols, resins, coatings, and pigments, and in the pharmaceutical and food industries, in the context of biorefineries, its main applications are the generation of intermediate chemical products such as 1,4-butanediol, tetrahydrofuran, 2-pyrrolidine, or maleic acid. Furthermore, the potential application of succinic acid and its derivatives for the manufacture of biodegradable polymers should also be mentioned [4–6].

Currently, the production volume of succinic acid through the biological route has already exceeded that generated by traditional chemical production. In addition, this growth is expected to continue in the coming years, so that the biotechnological production of this compound will go from generating a market value of USD 170 million in 2020 up to USD 2.22 billion by 2026 [7].

Among the microorganisms that produce succinic acid, it is worth highlighting *Actinobacillus succinogenes*, isolated from the rumen, as one of the most promising bacteria, since this acid is a final product during its anaerobic metabolism [8,9].

Numerous studies have been carried out focused on determining the influence of different variables on the production process of succinic acid using this microorganism as a biocatalyst. Despite the initial concentration of substrate being one of the most studied operating conditions, there is not yet a scientific consensus on its optimal value. For example, in the experiments carried out by Luthfi et al. [10] and Salvachúa et al. [11], the highest yields were achieved in fermentations starting from a concentration of 60 g L^{-1} of glucose, while Ferone et al. [12] achieved the best results when this concentration was reduced to 40 g L^{-1}. These latter authors also observed that low concentrations of xylose, around 5 g L^{-1}, increased the yield of succinic generation. However, Pateraki et al. [13], when using a mixture of sugars rich in xylose as a carbon source, achieved better results at a total sugar concentration of 32.5 g L^{-1}.

Another of the most studied parameters is the CO_2 source. Diverse authors have employed different carbonates ($NaHCO_3$, K_2CO_3, $CaCO_3$, or $MgCO_3$) at several concentrations [14,15], comparing them with pure gaseous CO_2 or biogas at different partial pressures [16–19], and even maximizing their solubility by increasing the pressure in the reactor [20,21]. Other studies have focused on cell status and on the mode of operation, performing experiments with free, immobilized cells or resting cells in batch, repeated batch, fed-batch, and continuous mode operations [22–28]. It is worth noting the work of Kim et al. [23], who carried out a continuous fermentation of recycled cells to maximize the biocatalytic activity, achieving a productivity of 3.86 g L^{-1} h^{-1} of succinic acid. However, the yields and productivities are especially high when working with immobilized cells, operating both in repeated batch. For example, Cao et al. [27] increased the production of succinic acid by approximately 50% compared to the batch operation. Furthermore, in continuous mode, Ercole et al. [25] produced 36.5 g L^{-1} h^{-1} of succinic acid with cells entrapped in alginate beads.

However, there is a lack of knowledge on other conditions that also affect the fermentation process with this microorganism, such as the initial biomass concentration or the agitation speed. In addition, until now, studies focused on the nitrogen source are scarce and the impact that this variable may have on production has not been fully explored [16,29,30]. It is worth noting the work of Tan et al. [31], who compared the use of 15 g L^{-1} of yeast extract (YE) and corn steep liquor (CSL), achieving a succinic acid amount 3.7% lower than using yeast extract as the nitrogen source and reducing by a fifth the costs associated with the nitrogen source. Xi et al. [32] studied the effect of using CSL at different initial concentrations, obtaining similar yields in fermentations with CSL and YE as long as the amount of CSL doubled that of YE. Jiang et al. [33] successfully replaced the yeast extract with a spent brewer's yeast hydrolysate with vitamin supplements.

Besides the many studies and proofs of concept published for the development of biorefinery processes, the main drawback of most of the proposed concepts is their viability at industrial scale. To carry out the scaling of a fermentation process, it is essential to develop kinetic models with equations capable of predicting the behavior of the species involved throughout production. These models allow an adequate selection of the type of operation, as well as the optimal design and operation of the bioreactor. In addition, they are very useful for the implementation of the control system and the performance of techno-economic studies [7,34].

However, the state-of-the-art research shows that the kinetic models developed on succinic acid production are based on results of fermentations carried out at different initial

concentrations of substrate. Therefore, as these studies are empirical in nature, they are only valid for the particular experimental conditions tested. Furthermore, there are no kinetic studies taking into account the effects of other operating conditions that are as influential as the concentration of the main carbon source. For these reasons, this work goes beyond the state-of-the-art research, using a novel approach to accomplish an exhaustive study of the influence exerted by diverse key operation variables—the initial concentration of biomass, the speed of agitation, and the concentration of yeast extract—in a batch production of succinic acid from glucose through the action of *A. succinogenes*. The developed new kinetic model is simple but capable of predicting the evolution of the concentration of biomass, glucose, succinic acid, and by-products for each of the experiments, and it was also applied to the production stages of a fed-batch fermentation.

2. Materials and Methods

2.1. Microorganism

Actinobacillus succinogenes DSM 22257, supplied by the German Collection of Microorganisms and Cell Cultures GmbH, was used in all experiments.

2.2. Culture Media

For storage, a 1:1 v/v glycerol/Tryptic Soy Broth (TSB) mixture was used [15,23,35] and for its subsequent reactivation, only TSB was used. TSB composition was (in grams per liter): 17 tryptone, 3 soytone, 2.5 glucose, 5 NaCl, 2.5 K_2HPO_4.

The medium for inoculum preparation as well as for the production reactor was the same [36] (in grams per liter): 3 K_2HPO_4, 0.43 $MgCl_2$.6H2O, 0.2 $CaCl_2$, 1 NaCl, 40 glucose, 2.5/5/7.5/10 yeast extract. In the case of the inoculum, 40 g L^{-1} of $NaHCO_3$ was added as a CO_2 source and for pH control. Carbon and nitrogen sources were autoclaved separately.

2.3. Cultivation Conditions

First, the stored cells were thawed at −80 °C and injected into bottles with 60 mL of TSB, which were previously purged with N_2 for 2 min at a flow rate of 1 L min^{-1}. After incubating the bottles at 37 °C and 200 rpm for 24 h, the inoculum was grown under the same conditions, but using, in this case, the production medium.

The study of the influence of operating conditions was carried out in a 2 L stirred tank bioreactor (STBR) BIOSTAT B-Plus (Sartorius AG, Göttingen, Germany) with a working volume of 1 L. In all runs, 0.1 vvm CO_2 was bubbled into the broth while operation was performed at 37 °C and a pH of 6.8 controlled by automatic addition of 5 M NaOH. The production medium was (in grams per liter): 3 K_2HPO_4, 0.43 $MgCl_2.6H_2O$, 0.2 $CaCl_2$, 1 NaCl, 40 glucose, 2.5/5/7.5/10 yeast extract. Experiments were carried out at different stirring speeds: 150, 200, 250, and 300 rpm, as well as at three different initial biomass concentrations: 0.05, 0.075, and 0.1 g L^{-1}.

In the fed-batch-type operation, the fermentation was carried out in the same reactor and with the same culture medium as in the batch type operations, working at 37 °C, 300 rpm, and pH 6.8 (5M NaOH), with an initial biomass concentration of 0.05 g L^{-1} and a yeast extract concentration of 10 g L^{-1}. After the first stage, a concentrated glucose solution was fed at the start of each of the following stages.

2.4. Analytical Methods

A spectrophotometer (Shimadzu UV-vis spectrophotometer UV-1603) was used to measure the biomass concentration, obtaining optical density data at 600 nm.

Glucose, succinic acid, and by-products (formic and acetic acids) were quantified by high-performance liquid chromatography (HPLC) (Agilent Technologies 100 series). The column employed for this analysis was a REZEX ROA-Monosaccharide H+ (8%) column (300 × 7.8 mm, Phenomenex, Torrance, CA, USA) at 80 °C pumping a H_2SO_4 5 mM solution as mobile phase at a flow rate of 0.5 mL min^{-1}. The refraction index detector worked at 55 °C.

3. Results and Discussion

The results for succinic acid titer (C_{SA}), yield with respect to the initial concentration of the carbon source (Y_{SA}), productivity (P_{SA}), and selectivity (S_{SA}) of the batch experiments carried out under different conditions of CO_2 flow, agitation speed, and yeast extract and initial biomass concentrations are shown in Table 1.

Table 1. Summary of succinic acid titers, yields, productivities, and selectivity values under different operational conditions. The first run is the reference one.

Run	Type of Operation	$C_{biomass}$ ($g \cdot L^{-1}$)	Agitation (rpm)	CO_2 Flow ($L \cdot min^{-1}$)	C_{YE} ($g \cdot L^{-1}$)	C_{SA} ($g \cdot L^{-1}$)	Y_{SA} ($g \cdot g^{-1}$)	P_{SA} ($g \cdot L^{-1} \cdot h^{-1}$)	S_{SA} ($g \cdot g^{-1}$)
1	Batch	0.05	300	0.1	10	27.4	0.68	0.83	0.62
2	Batch	0.075	300	0.1	10	28.5	0.71	0.96	0.64
3	Batch	0.1	300	0.1	10	28.3	0.70	0.76	0.66
4	Batch	0.05	300	0.5	10	27.6	0.69	0.84	0.63
5	Batch	0.05	300	1	10	26.1	0.65	0.81	0.63
6	Batch	0.05	150	0.1	10	23.6	0.59	0.72	0.61
7	Batch	0.05	200	0.1	10	26.4	0.66	0.78	0.62
8	Batch	0.05	250	0.1	10	28.5	0.71	0.84	0.62
9	Batch	0.05	300	0.1	2.5	23.8	0.59	0.48	0.68
10	Batch	0.05	300	0.1	5	26.8	0.66	0.53	0.66
11	Batch	0.05	300	0.1	7.5	28.9	0.72	0.58	0.64
12	Fed-batch	0.05	300	0.1	10	39.7	0.67	0.72	0.69

It should be noted that the first experiment was taken as the reference one, because its operating conditions are those that can be considered standard or intermediate between those more used in batch-type fermentation for the production of succinic acid using *A. succinogenes* as a biocatalyst [10,11,13,23,26,37–42].

In runs 1, 2, and 3, the initial concentration of biomass is a factor whose variation mainly affected the productivity of the process. It was observed that at an initial concentration of 0.075 g L^{-1}, the intermediate value of those studied, the productivity of succinic acid reaches a maximum value of 0.96 g L^{-1} h^{-1}. However, if the cell concentration is further increased to 0.1 g L^{-1}, productivity is reduced by 21%. In addition, a slight increase in selectivity was observed at higher initial amounts of biomass in the reactor.

Until now, an optimal initial concentration of biomass in the production process of succinic acid by *A. succinogenes* has not been determined in the literature, although a couple of studies have been carried out in which an attempt has been made to determine the optimum inoculum size, arriving at certainly different conclusions. On the one hand, Wan et al. [43] observed that an inoculum size of 10% compared to 2% or 5% led to higher yields of succinic acid, which can be justified by relating a greater amount of cell density with reduced latency time. However, Anwar et al. [44] studied the effect of the inoculum size in a succinic acid production process through simultaneous saccharification and fermentation, concluding that increasing the inoculum size from 5% to 15% reduced the final concentration of succinic acid generated by 50%. The latter authors attributed this reduction in yield to the strong competition for nutrients that occurs in the culture broth when cell density is very high.

Considering these observations and the results of this work, it seems that an initial biomass concentration of an intermediate value is required that, on the one hand, is sufficient to avoid long latency times, but, on the other hand, does not lead to too rapid consumption of the carbon and nitrogen source. Other authors have also observed, in studies carried out in different fermentation processes, that excessive initial amounts of

biomass also lead to inhibitions by the product and accumulation of metabolites, which considerably impair the performance of the process [45,46].

However, these reflections do not seem to be sufficient to justify the increase in selectivity that occurs at higher initial biomass concentrations. This reduction in the number of by-products seems to be associated with possible variations in metabolism. To clarify this matter, it would be necessary to conduct metabolic analysis by carrying out fermentations with *A. succinogenes* under different inoculum size conditions. This was undertaken by Din et al. [47] with *Saccharomyces cerevisiae*, who observed large changes in glucose metabolism intermediates, amino acids, and metabolites related to the structure of the cell membrane by modifying the inoculum size.

In runs 1, 4, and 5, the CO_2 gas flow rate was increased from 0.1 to 1 L min^{-1}, values that include those that can be found in the literature on succinic acid production [10,11,13,23,26,37–42]. In this study, no significant variations in yield, productivity, or generation of by-products were observed in the range of flows studied. Taking into account the work of Xi et al. [17] and Zou et al. [18], who observed differences in the production of succinic acid working with mixtures of N_2 and CO_2 until reaching saturation of the latter gas, it is concluded that, as in the present work, fermentations with an excess of CO_2 do not favor the deviation of the metabolism towards the generation of succinic acid to the detriment of other metabolites. This means that most of the studies on succinic acid production by *A. succinogenes* published to date were carried out under conditions that involve higher economic costs and do not offer any additional advantage.

The increase in the agitation speed between 150 and 300 rpm, the range of values typically used in the literature [10,11,13,23,37–41,48,49], of runs 1, 6, 7, and 8, shows a considerable improvement in performance and productivity at high stirring values, reaching the best results at a stirring speed of 250 rpm. This operating condition is one of the factors with the greatest impact on the transfer of gases in liquid media, decreasing mixing time and improving mass and heat transfer rates [50,51]. Therefore, it can be deduced that the stirring values of 150 and 200 rpm are insufficient to achieve an adequate transfer of CO_2 in the culture broth. Taking this phenomenon into account, it could be deduced that the higher the stirring speed, the greater the generation of succinic acid; however, excessive shearing forces can lead to cell damage and, as a consequence, to the reduction in the process performance [52]. This seemed to happen in the run performed at 300 rpm, in which the effect of hydrodynamic stress appeared to be reflected.

In runs 1, 9, 10, and 11, the effect of the initial concentration of the nitrogen source in the culture medium was compared. As the YE concentration increased from 2.5 to 10 g L^{-1}, so did the succinic acid productivity. However, the yield of succinic acid with respect to the initial concentration of the carbon source reached its maximum in the fermentations carried out with 7.5 g L^{-1} of YE. However, it should be noted that Jiang et al. [33] achieved their maximum yield of succinic acid at around 20 g L^{-1} of YE, although they do not provide data on productivity or generation of by-products to be able to make a more detailed comparison with the present study. On the other hand, a tendency to improve selectivity was observed as the quantity of the nitrogen source decreased, which agrees with the conclusions of Ventrone et al [53], who observed that high C:N ratios lead to a reduction in by-product formation. In fact, in operations in the absence of a nitrogen source, with resting cells, Escanciano et al. [28] reduced the generation of by-products by 27.5% compared to the equivalent operation with cells in a state of growth, that is, in the presence of a nitrogen source.

The fed-batch run (run 12) reduced the number of by-products generated compared to the reference experiment, increasing the selectivity from 0.62 g g^{-1} (run 1) to 0.69 g g^{-1} (run 12). During their fed-batch production of succinic acid from citrus peel waste, Patsalou et al. [54] observed that the by-products were produced mainly in the first 24 h in a fermentation lasting more than 60 h, while the succinate continued to be generated throughout the entire process. In addition, these authors obtained a marked drop in productivity, as occurred in the present work, which decreased from 0.83 g L^{-1} h^{-1} in the batch operation type to 0.72 g L^{-1} h^{-1} in the fed-batch fermentation. The loss in the

production rate of succinic acid in this type of operation is a conclusion shared by more authors; for example, Kanchanasuta et al. [55] also saw a depletion in yield, a trend that was also observed in this work, although in a less pronounced way. Taking into account that succinic acid production appears to be favored in a non-growth steady state [56,57] and that nutrient depletion does not appear to be an obstacle to succinic acid production when there is already a high biomass concentration [28], it seems that the main problem caused by this type of operation is the excessive accumulation of metabolites, which can generate cell damage and strong inhibitions by product [7,13,37,58,59].

3.1. Development of a Simple Kinetic Model

For the development of the kinetic model, a reaction scheme was proposed based on the time course of the biomass, substrate (glucose), and fermentation products (succinic, acetic, and formic acids) of the reference experiment (run 1), whose evolution throughout over time is shown in Figure 1A. It is observed that the biomass grows until reaching its maximum at 10 h of fermentation; however, both succinic acid and by-products continue to increase over time until the carbon source is exhausted around 33 h, indicating that production is not associated with growth. However, the rate of formation of acetic and formic acids slows down after approximately 20 h of fermentation, while the rate of production of succinic acid only suffers a slight reduction in the last hours of the process. In addition, although a greater amount of acetic acid is produced than formic acid, both compounds follow the same growth trend, which is why it was decided to combine both acids, as shown in Figure 1B, with the aim of proposing a model with the metabolite "by-products" (BPs) that allows further reduction in the number of kinetic parameters and the development of a more useful model from the point of view of chemical engineering.

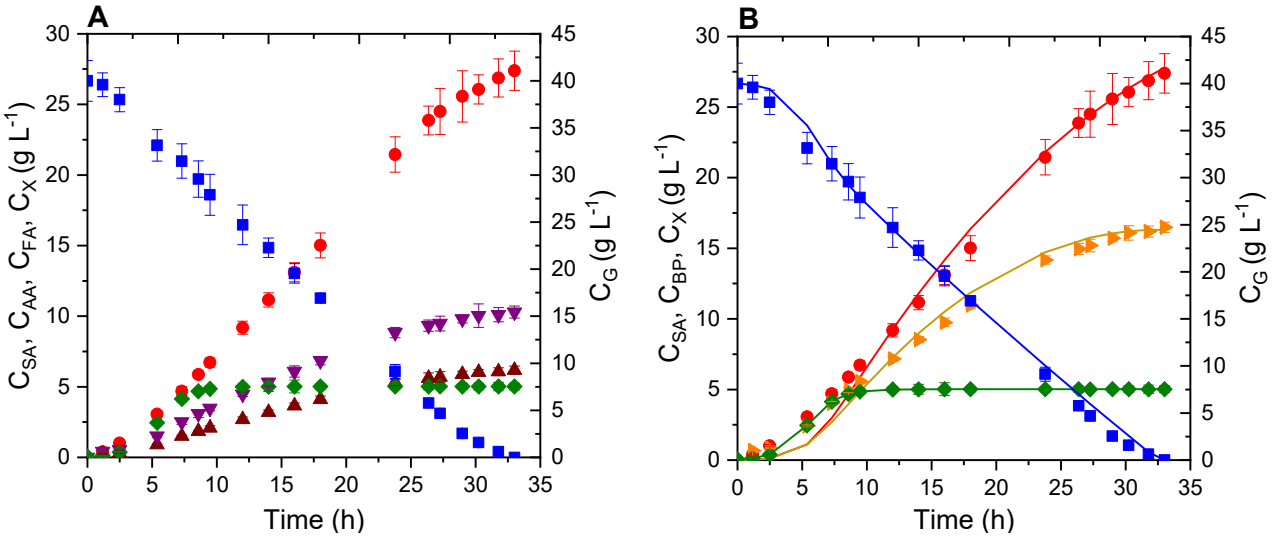

Figure 1. Evolution of the concentration of succinic acid (SA), acetic acid (AA), formic acid (FA), biomass (X), and glucose (G) over time in run 1 (reference). (**A**) representation of AA and FA concentrations separately. (**B**) combining of AA and FA concentrations into by-product (BP) concentration and kinetic model prediction. Data points: SA (●), G (■), AA (▼), FA (▲), X (♦), BP (►), model predictions shown as lines.

Based on these data, a very simple reaction scheme based only on Equations (1)–(3) is proposed. It is an unstructured non-segregated model, that is, the microorganism is considered as a single component, the biomass. This scheme is made up of a first reaction (r_1) for the consumption of glucose (S) for the generation of biomass, a second reaction (r_2) for the generation of succinic acid (P) and by-products (BPs), and a last reaction (r_3) for the independent generation of by-products. Their corresponding rates are shown in Equations (4)–(6), while the consumption and formation rates (R_j) of compounds 'j'

are described in Equations (7)–(10). Therefore, the biomass has a growth rate based on the logistic equation, whose kinetic parameters are the specific growth rate (μ) and the maximum biomass concentration (C_{Xm}). The generation of succinic acid and by-products is governed by potential equations independently of the growth of the microorganism in a proportional way to their kinetic constants K_{p1} and K_{p2}. In addition, together with these parameters, the formation and consumption rates are also defined by the macroscopic yields $Y_{S/X}$, $Y_{S/P1}$, $Y_{S/BP,}$ and $Y_{S/P2}$.

Reaction network

$$Y_{S/X}S \xrightarrow{r_1} X \quad (1)$$

$$Y_{S/P1}S \xrightarrow{r_2} P + Y_{S/BP}\cdot BP \quad (2)$$

$$Y_{S/P2}S \xrightarrow{r_3} BP \quad (3)$$

Reaction rates

$$r_1 = \mu\cdot C_X\cdot\left(1 - \frac{C_X}{C_{Xm}}\right) \quad (4)$$

$$r_2 = k_{P1}\cdot C_S\cdot C_X \quad (5)$$

$$r_3 = k_{P2}\cdot C_S\cdot C_X \quad (6)$$

Production and consumption rates

$$R_S = \frac{dC_S}{dt} = -Y_{S/X}\cdot r_1 - Y_{S/P1}\cdot r_2 - Y_{S/P2}\cdot r_3 \quad (7)$$

$$R_P = \frac{dC_P}{dt} = r_2 \quad (8)$$

$$R_X = \frac{dC_X}{dt} = r_1 \quad (9)$$

$$R_{BP} = \frac{dC_{BP}}{dt} = Y_{S/BP}\cdot r_2 + r_3 \quad (10)$$

Applying this model, the calculation of its kinetic parameters (Table 2) was performed from the data of the reference experiment (run 1). The simulation of the evolution of the biomass, substrate, product, and by-products is shown in Figure 1B, together with the experimental data, obtaining an excellent fit despite the simplicity of the model and the reduced number of parameters. In addition, Table 3 presents the statistical parameters that reflect the goodness of fit. A value of Fisher's F (F_{95}) of 41,242 was obtained, much higher than its tabulated value at 95% (where 8.55 is the value of F tabulated at that probability) and a percentage of explained variation very close to 100% (99.5%). In addition, very low values were obtained in those parameters that should be as close to zero as possible, with a sum of squared residuals (SSR) of 6.47 and a residual mean squared error (RMSE) of 0.67.

Table 2. Summary of kinetic parameters under different operational conditions.

Run	Type of Operation	$C_{biomass}$ ($g \cdot L^{-1}$)	Agitation (rpm)	CO_2 Flow ($L \cdot min^{-1}$)	C_{YE} ($g \cdot L^{-1}$)	Cxm ± Error ($g_X \cdot L^{-1}$)	Kp_1 ± Error ($L \cdot g^{-1} \cdot h^{-1}$)	Kp_2 ± Error ($L \cdot g^{-1} \cdot h^{-1}$)	μ ± Error (h^{-1})	$Y_{S/P1}$ ± Error ($g \cdot g^{-1}$)	$Y_{S/P2}$ ± Error ($g \cdot g^{-1}$)	$Y_{S/BP}$ ± Error ($g \cdot g^{-1}$)	$Y_{S/X}$ ± Error ($g \cdot g^{-1}$)
1	Batch	0.05	300	0.1	10	5.02 ± 0.02	0.007 ± 0.001	0.019 ± 0.001	0.85 ± 0.04	0.24 ± 0.02	2.27 ± 0.22	1.10 ± 0.09	1.65 ± 0.15
2	Batch	0.075	300	0.1	10	5.81 ± 0.03	0.009 ± 0.001	0.013 ± 0.001	0.85 ± 0.03	0.24 ± 0.02	2.20 ± 0.19	1.08 ± 0.07	1.63 ± 0.16
3	Batch	0.1	300	0.1	10	6.79 ± 0.08	0.004 ± 0.000	0.008 ± 0.001	0.85 ± 0.02	0.24 ± 0.02	2.25 ± 0.19	1.09 ± 0.08	1.63 ± 0.16
4	Batch	0.05	300	0.5	10	5.07 ± 0.03	0.008 ± 0.001	0.018 ± 0.001	0.85 ± 0.01	0.25 ± 0.02	2.27 ± 0.22	1.09 ± 0.09	1.66 ± 0.16
5	Batch	0.05	300	1	10	5.07 ± 0.03	0.008 ± 0.001	0.018 ± 0.001	0.85 ± 0.01	0.25 ± 0.02	2.27 ± 0.22	1.09 ± 0.09	1.66 ± 0.16
6	Batch	0.05	150	0.1	10	5.09 ± 0.09	0.004 ± 0.001	0.019 ± 0.001	0.85 ± 0.04	0.26 ± 0.02	2.30 ± 0.21	1.10 ± 0.08	1.56 ± 0.13
7	Batch	0.05	200	0.1	10	5.05 ± 0.06	0.006 ± 0.001	0.018 ± 0.001	0.85 ± 0.06	0.26 ± 0.02	2.27 ± 0.18	1.04 ± 0.07	1.54 ± 0.11
8	Batch	0.05	250	0.1	10	5.00 ± 0.05	0.008 ± 0.001	0.018 ± 0.001	0.85 ± 0.05	0.24 ± 0.02	2.31 ± 0.18	1.07 ± 0.09	1.60 ± 0.13
9	Batch	0.05	300	0.1	2.5	2.40 ± 0.02	0.008 ± 0.001	0.022 ± 0.001	0.85 ± 0.05	0.24 ± 0.02	1.45 ± 0.10	0.86 ± 0.01	0.98 ± 0.02
10	Batch	0.05	300	0.1	5	5.01 ± 0.03	0.004 ± 0.001	0.011 ± 0.001	0.85 ± 0.03	0.25 ± 0.01	1.76 ± 0.11	0.95 ± 0.02	1.65 ± 0.02
11	Batch	0.05	300	0.1	7.5	5.08 ± 0.03	0.005 ± 0.001	0.014 ± 0.001	0.85 ± 0.04	0.24 ± 0.01	2.06 ± 0.17	1.00 ± 0.04	1.65 ± 0.04
12	Fed-batch cycle 1	0.05	300	0.1	10	5.02 ± 0.02	0.007 ± 0.001	0.019 ± 0.001	0.85 ± 0.04	0.24 ± 0.02	2.27 ± 0.22	1.10 ± 0.09	1.65 ± 0.15
12	Fed-batch cycle 1	0.05	300	0.1	10	5.02 ± 0.02	0.007 ± 0.001	0.019 ± 0.001	0.85 ± 0.04	0.24 ± 0.02	2.27 ± 0.22	0.59 ± 0.02	1.65 ± 0.15
12	Fed-batch cycle 1	0.05	300	0.1	10	5.02 ± 0.02	0.004 ± 0.001	0.005 ± 0.000	0.85 ± 0.04	0.24 ± 0.02	2.27 ± 0.22	0.37 ± 0.01	1.65 ± 0.15

Table 3. Summary of statistical parameters under different operational conditions.

Run	Type of Operation	$C_{biomass}$ ($g \cdot L^{-1}$)	Agitation (rpm)	CO_2 Flow ($L \cdot min^{-1}$)	C_{YE} ($g \cdot L^{-1}$)	F_{95}	RMSE	SSR	VE %
1 REF.	Batch	0.05	300	0.1	10	41,242	0.67	6.47	99.5
2	Batch	0.075	300	0.1	10	13,660	1.09	11.26	98.2
3	Batch	0.1	300	0.1	10	40,640	1.00	10.04	98.7
4	Batch	0.05	300	0.5	10	8457	1.14	12.17	98.5
5	Batch	0.05	300	1	10	8457	1.14	12.17	98.5
6	Batch	0.05	150	0.1	10	19,384	0.92	9.01	99.0
7	Batch	0.05	200	0.1	10	19,684	1.03	9.99	98.6
8	Batch	0.05	250	0.1	10	11,751	1.07	11.70	98.5
9	Batch	0.05	300	0.1	2.5	5037	1.19	14.29	97.3
10	Batch	0.05	300	0.1	5	22,441	1.10	11.57	98.9
11	Batch	0.05	300	0.1	7.5	17,270	1.00	6.32	98.9
12	Fed-batch cycle 1	0.05	300	0.1	10	41,242	0.67	6.47	99.5
12	Fed-batch cycle 1	0.05	300	0.1	10	13,512	1.05	8.97	98.4
12	Fed-batch cycle 1	0.05	300	0.1	10	24,175	0.95	6.09	99.6

After verifying the validity of this model with the reference experiment, it was applied to all the other fermentations carried out under different operating conditions (runs 2–12), with the aims of verifying its robustness, studying the variation in the parameters, and knowing in greater depth the real impact of each of the operating conditions in the succinic acid production process. The estimated kinetic parameters for each of the experiments are shown in Table 2, while the statistical parameters are presented in Table 3.

Until now, the kinetic models of succinic acid production by *A. succinogenes* have been scarce and of the unstructured non-secreted type. In addition, they are usually limited to the study of the rate of biomass formation, leaving aside the evolution of the metabolites present in the broth [12,60,61], although some authors such as Pateraki et al. [13] Lin et al. [37], and Vlysidis et al. [58] have also focused on the carbon source, as well as on the evolution of succinic acid and the generated by-products. Lin et al [37] proposed a biomass growth equation from glucose based on a combination of the Monod equation and the Luong equations for substrate and product inhibition. Vlysidis et al. [58] and Pateraki et al. [13] coincided in using the same combination of equations but used a Haldane–Andrews-type substrate inhibition term. Despite its low value, in all these works Pirt's maintenance coefficient is taken into account when estimating the consumption of the carbon source. In addition, they have in common that they resort to the Luedeking–Piret expression to predict the generation of succinic acid and by-products [7].

Although, on the one hand, they are very complete models that can provide a large amount of information about the fermentation process, on the other hand, the fact that they are precisely made up of long equations with a large number of parameters is impractical when, for example, carrying out an industrial scaling process, designing a control system, or carrying out a techno-economic analysis. In these circumstances, as demonstrated in this work, it is also possible to predict the evolution of the biomass and the same quantity of metabolites with a simpler system of equations based on the previous study of the relationship between the species present in the culture broth.

3.2. Kinetic Study Based on the Initial Biomass Concentration

After applying the kinetic model to runs 1, 2, and 3, a good adjustment to the experimental data was achieved, as shown by the corresponding statistical parameters in Table 3. In these experiments, carried out at increasing initial biomass concentrations, it was observed that most of the estimated kinetic parameters did not suffer variations despite the modification of this operating condition (Table 2). However, three parameters of the model experienced considerable modifications and their variation is represented in Figure 2.

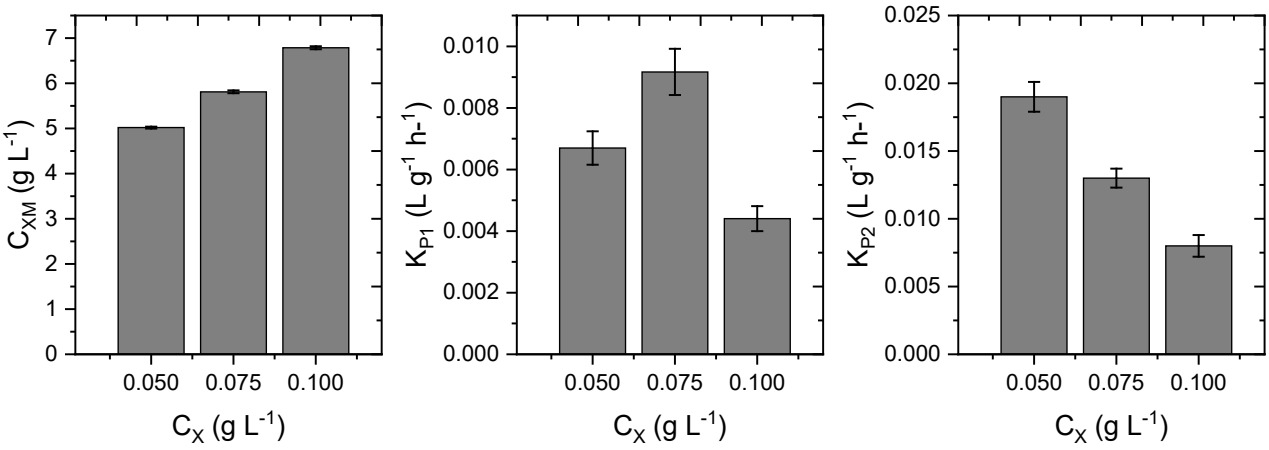

Figure 2. Kinetic parameters that are modified as a function of the initial biomass concentration.

On the one hand, the C_{xm} parameter shows that the increase in the initial biomass also leads to a higher maximum concentration of biomass in the culture broth once the stationary phase of its growth has been reached, with a practically linear trend, as expected. On the other hand, a drop in the kinetic constant of the by-product formation reaction (K_{P2}) is also observed, independent of that of succinic acid as the cell density increases in the broth, which agrees with the upward trend in selectivity previously discussed. Finally, the correlation between succinic acid productivity (Table 1) and the kinetic constant of the succinic acid formation equation (K_{P1}) is observed, reaching its maximum at an initial biomass concentration of 0.075 g L^{-1}.

3.3. Kinetic Study Based on the CO_2 Flow

As discussed in Section 3.1, despite modifying the CO_2 flow between values typically used in the literature [10,11,13,23,37–41,48,49], no variations were observed in the yield, productivity, or selectivity of the process (Table 1). The reason for this is that it seems that the excess of this gas does not propitiate the displacement of the metabolic route towards the formation of succinic acid. This deduction was confirmed with the application of the kinetic model to runs 1, 4, and 5, which enabled a simultaneous estimation of the three experiments without variation in the kinetic parameters (Table 2) and adequate goodness of fit (Table 3). Figure 3 shows the evolution of the experimental data of biomass, glucose, succinic acid, and by-products over time of runs 1, 4, and 5, together with the representation of the prediction of the evolution of their concentrations made on the whole.

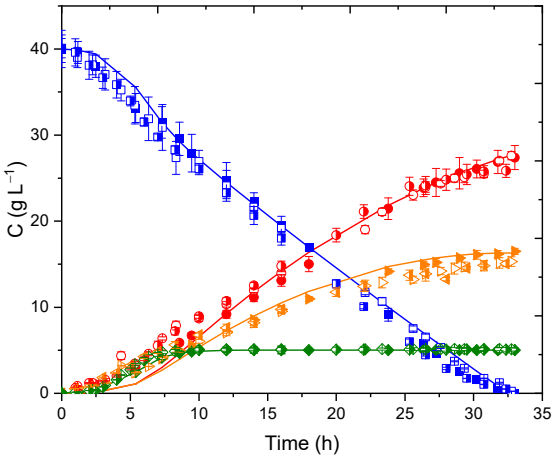

Figure 3. Kinetic model of the evolution of succinic acid (SA), by-products (BPs), biomass (X), and glucose concentrations (G) over time depending on CO_2 flow (0.1, 0.5, 1 L min^{-1}). Data points: SA (●), G (■), X (♦), BP (►), 0.1 L min^{-1} close symbol, 0.5 L min^{-1} open symbol, 1 L min^{-1} half open symbol, model predictions shown as lines.

3.4. Kinetic Study Based on the Stirring Speed

Agitation is an operating condition whose increase favors the transfer of gas in the culture broth and the homogeneity of the compounds, in turn improving the productivity and yield of succinic acid (Table 1); in this case, the maximum reached was 250 rpm. However, above this speed, the cells seem to suffer damage, negatively affecting the development of the process. This behavior is reflected exactly in the parameters of the kinetic model, showing growth in the kinetic constant of the reaction for the formation of succinic acid (K_{P1}) until reaching a maximum at 250 rpm, and then a considerable reduction at 300 rpm, as shown in Figure 4.

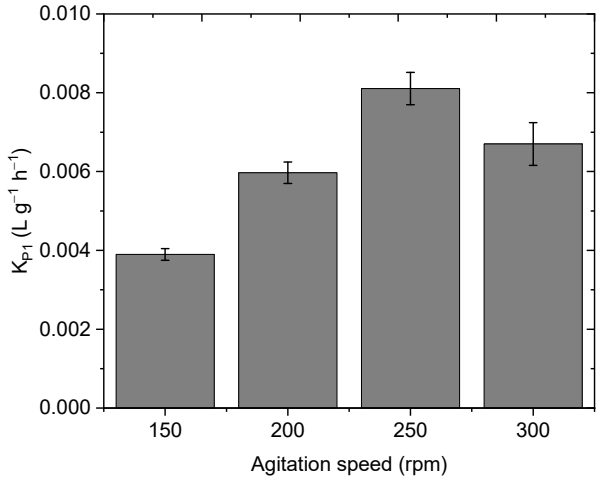

Figure 4. Kinetic parameters that are modified as a function of the stirring speed.

Despite the fact that, in this case, it was possible to observe an increase in productivity and possible cell damage, it should be noted that these conclusions differ from those of other authors such as Bevilaqua et al. [62], who did not observe significant variations in the performance of the process despite increasing the agitation up to 300 rpm, using the same microorganism as a biocatalyst although using hydrochloric hydrolysates of RH as the substrate instead of glucose. Gonzales et al [30] also studied the effect of stirring speed, performing experiments between 100 and 300 rpm. They only observed variations in the biomass concentration and determined that the optimum was to operate at low agitation speeds (100 rpm). These differences in the conclusions clearly open a door to the extension

of the study of the influence of this operating condition, whose real impact seems yet to be determined.

3.5. Kinetic Study Based on the Yeast Extract Concentration

As discussed in Section 3.1, the concentration of yeast extract is a variable that has a great impact on the performance and productivity of the process, so that as the nitrogen source increases, higher productivity is achieved. However, the succinic yield peak was not reached at the maximum concentration studied (10 g L^{-1}), but at 7.5 g L^{-1}. Figure 5 shows that the kinetic estimations made in runs 1, 9, 10, and 11 led to the variation in six kinetic parameters. First, in the experiment at 2.5 g L^{-1} of YE (run 9), the values of the maximum biomass concentration (C_{xm}) and the macroscopic yield of biomass production ($Y_{S/X}$) were approximately half of those corresponding to all other fermentations carried out at higher concentrations of the nitrogen source (runs 1, 10, 11). Therefore, taking into account that there was not a great decrease in yield between run 9 and the rest of the experiments, it can be deduced that the amount of succinic acid and by-products generated per gram of biomass is much higher at 2.5 g L^{-1} of YE than in the experiments carried out at higher concentrations of YE, which results in an increase in the values of K_{P1} and K_{P2}.

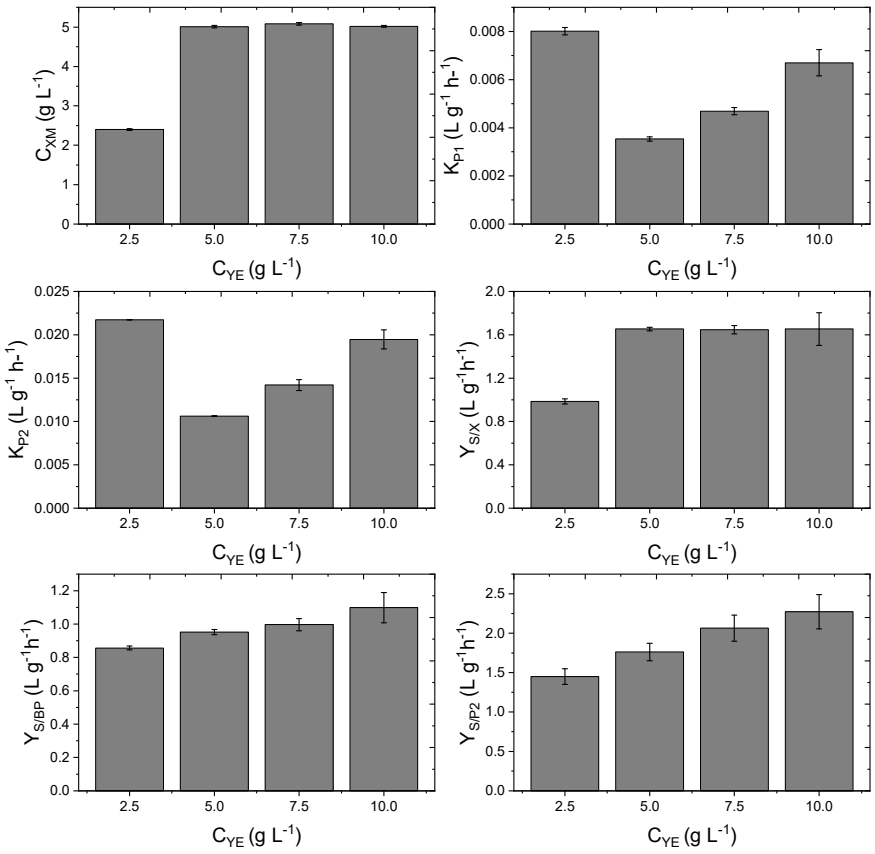

Figure 5. Kinetic parameters that were modified as a function of the yeast extract concentration.

It can also be observed that starting from 5 g L^{-1} of YE, the increase in the nitrogen source leads to the same increasing evolution of K_{P1} and K_{P2} as that of productivity. Despite this, an increase in the yield parameters related to the by-products ($Y_{S/BP}$, $Y_{S/P2}$) was observed as the initial concentration of YE increased, both in the simultaneous generation reaction of succinic acid and by-products (r_2), as well as in the isolated by-product generation reaction (r_3). This is consistent with the data presented in Table 1, that is, with the increase in selectivity with the decrease in the initial amount of YE.

3.6. Kinetic Estimation of the Stages of a Fed-Batch Type Operation

To check the robustness of the model for long fermentation times, a fed-batch fermentation (run 12) was performed. Kinetic estimates were made for each of the three stages, and simulations of the evolution of the biomass, substrate, product, and by-products over time were performed from maximum and minimum values of the kinetic parameters within the confidence interval. Figure 6 shows the experimental concentrations of biomass and metabolites over time, along with the prediction lines of the kinetic model and those simulated from the confidence interval. The yield values of succinic acid with respect to consumed glucose ($Y_{S/Gc}$) are also included, as well as the productivity and selectivity of succinic acid in the three stages. Figure 7 shows the kinetic parameters that underwent modifications throughout the three stages of the process.

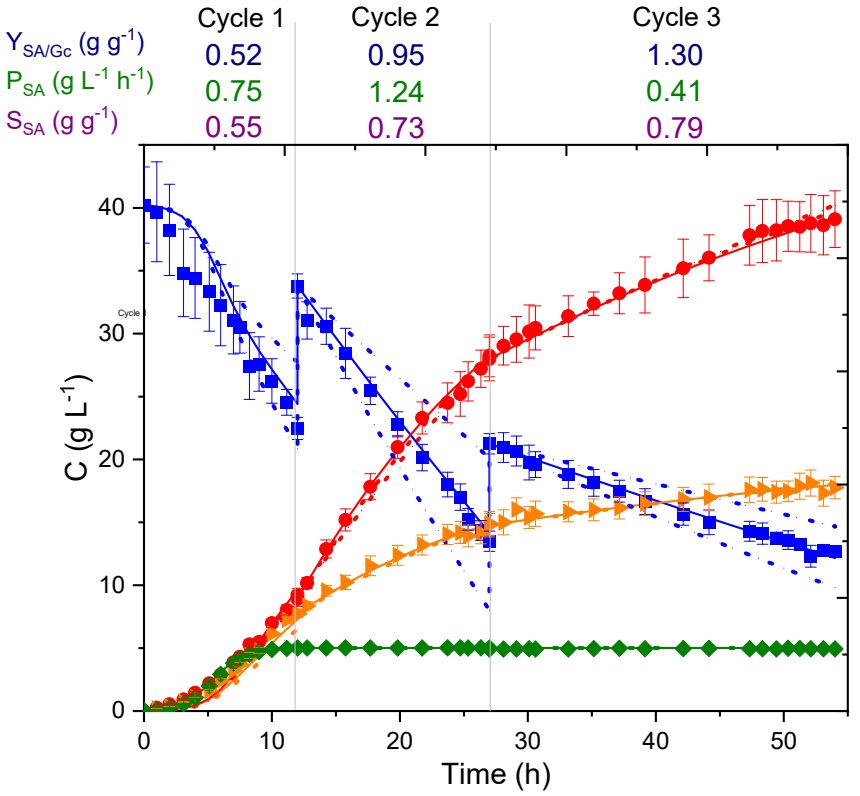

Figure 6. Evolution of the concentration of succinic acid (SA), by-products (BPs), biomass (X) and glucose (G) over time in run 11 (fed-batch). $Y_{SA/Gc}$: yield as a function of glucose consumed, P_{SA}: succinic acid productivity, S_{SA}: selectivity. Data points: SA (●), G (■), X (♦), BP (►), model estimations and simulations shown as lines.

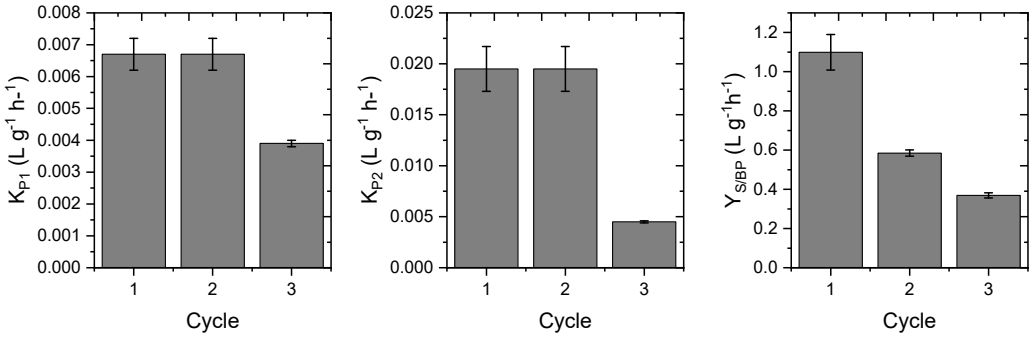

Figure 7. Kinetic parameters that were modified as a function of the fed-batch cycle.

It should be noted that, in the second stage, in line with the increase in selectivity with respect to the previous stage, there was a decrease in the yield parameter towards by-products (YS/BP) of reaction 2. Therefore, due to a greater use of glucose in succinic acid instead of by-products, it is logical that there is an increase in yield and productivity in this intermediate stage, without the need to alter any other kinetic parameter of the model.

Due to the increase in the selectivity of the third stage with respect to the previous stages, the macroscopic performance regarding by-products of reaction 2 ($Y_{S/BP}$) suffers a considerable reduction. However, the drop in succinic acid productivity is only reflected in the decrease in the kinetic constants of reactions 2 and 3 (K_{P1} and K_{P2}), parameters that decrease the reaction rate in a directly proportional manner. The simulation of the first cycle of run 12 could be carried out with the parameters estimated for run 1, since they share the same operating conditions.

4. Conclusions

In this work, an exhaustive study of critical variables in the bioproduction process of succinic acid by *A. succinogenes* was carried out to determine the effect of variables such as the initial concentration of biomass, the agitation speed, the concentration of yeast extract, and the CO_2 flow. A simple but robust kinetic model was developed which, unlike the models currently found in the literature, is capable of predicting the evolution of glucose, succinic acid, by-products, and biomass with few kinetic parameters. Its application to a reference run allowed verification of the goodness of fit, obtaining high values of F_{95} (41,242) and VE (99.5%), and values of RMSE and SSR close to zero. Subsequently, its validity was also demonstrated by estimating the evolution of metabolites in experiments in which the initial biomass concentration, the yeast extract concentration, agitation, and CO_2 flow were modified. Until now, these variables have not been used in the literature for the development of a succinic acid production kinetic model. Finally, the model was applied to a fed-batch type operation, performing simulations based on the confidence intervals of the estimated parameters for each of the stages. In this way, it was possible to develop and validate a model, having significant robustness and simplicity, which is very useful from the point of view of chemical engineering for the scaling of the process, the design of a control system, or the performance of techno-economic analyses.

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